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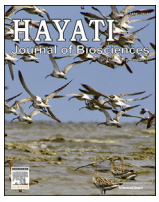

Original research article

Diversity of Protease-Producing *Bacillus* spp. From Fresh Indonesian Tempeh Based on 16S rRNA Gene Sequence

Tati Barus,[1*] Linda Wati,[1] Melani,[1] Antonius Suwanto,[2] Yogiara[1]

[1] *Atma Jaya Catholic University of Indonesia, Jakarta 12930, Indonesia.*
[2] *Bogor Agricultural University, Bogor 16680, West Java, Indonesia.*

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ABSTRACT

Tempeh is a type of traditional fermented food in Indonesia. The fermentation can be performed by *Rhizopus microsporus* as a main microorganism. However, *Bacillus* spp. is found in abundance in tempeh production. Nevertheless, information regarding the diversity of *Bacillus* spp. in tempeh production has not been reported yet. Therefore, the aim of this investigation was to study the genetic diversity of *Bacillus* spp. in tempeh production based on the 16S ribosomal RNA sequence. In this study, about 22 of 24 fresh tempeh from Jakarta, Bogor, and Tangerang were used. A total of 52 protease-producing *Bacillus* spp. isolates were obtained. Based on 16S ribosomal RNA results, all 52 isolates were identified to be similar to *B. pumilus, B. subtilis, B. megaterium, B. licheniformis, B. cereus, B. thuringiensis, B. amyloliquefaciens, Brevibacillus brevis*, and *Bacillus* sp. All the identified isolates were divided into two large clusters: 1) a cluster of *B. cereus, B. thuringiensis, Bacillus* sp., and *B. brevis* and 2) a cluster of *B. pumilus*, *B. subtilis, B. megaterium, B. licheniformis*, and *B. amyloliquefaciens*. Information about the *Bacillus* spp. role in determining the quality of tempeh has not been reported and this is a preliminary study of *Bacillus* spp. from tempeh.

1. Introduction

Tempeh is an Indonesian traditional fermented food made from soybeans which has many advantages. It has been reported that tempeh can prevent diarrhea (Sudigbia 1999) and anemia (Astuti 1999) because of increased iron availability during fermentation. Tempeh contains vitamin B12 (Keuth & Bisping 1994) and antioxidant compounds (Esaki *et al.* 1996). Vitamins and minerals in tempeh are higher than those in raw soybeans and this is most likely due to fermentation. Previous studies also reported that genistein, daidzein, and β-sitosterol which are found in tempeh can prevent cancer, cardiovascular diseases, and osteoporosis (Karyadi and Lukito 1996; Hermana *et al.* 1999; Dave *et al.* 2005). Phytic acid and trypsin (antinutritive factors) are found in small amounts in tempeh or after the fermentation of soybeans (Hong *et al.* 2004). Various beneficial factors of tempeh make it popular in several countries, including Japan, Netherland, and USA (Aderibigbe and Osegboun 2006), especially in the diet of vegetarians.

Fermentation of tempeh involves various microorganisms such as molds, yeasts, and bacteria. Common molds found in tempeh are *Rhizopus stolonifer*, *R. arrhizus*, *R. oryzae*, and *R. formosaensis*, while yeasts involved in tempeh fermentation are *Pichia burtonii* and *Candida didensiae* (Mulyowidarso *et al.* 1989). In addition, common bacteria found in tempeh include *Klebsiella pneumoniae* and *Bacillus* sp. (Barus *et al.* 2008).

Bacillus spp., especially *B. subtilis*, is the main bacteria found in soybean-based fermented foods. The role of *Bacillus* spp. in improving the quality of soybean-based fermented foods has been reported, such as in Thai Thua nao (Dajanta *et al.* 2009; Tangjitjaroenkun 2004), Indian Kinema (Sarkar *et al.* 2002), Korean Cheonggukjang (Kwon *et al.* 2009), Japanese natto (Hsu *et al.* 2009), and the African Dawadawa (Terlabie *et al.* 2006) and Soumbala (Sarkar *et al.* 2002). Thua nao fermented with *Bacillus* sp. B4 shows high proteolytic activity enabling effective release of soluble proteins (Dajanta *et al.* 2001). However, there is very limited

* Corresponding author. Faculty of Biotechnology, Atma Jaya Catholic University, Jl. Jenderal Sudirman No. 51, Jakarta 12930, Indonesia.
E-mail addresses: tati.barus@atmajaya.ac.id (T. Barus), linda_cutesquireel@yahoo.com (L. Wati), melani.mustafa@hotmail.com (Melani), antoniussuwanto@gmail.com (A. Suwanto), yogiara@yahoo.com (Yogiara).

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information on the diversity of *Bacillus* strains present in conventionally prepared tempeh, especially, the *Bacillus* spp. The enzyme protease produced by the *Bacillus* spp. is necessary to digest the soy protein which is otherwise easily exploited by our body.

A range of molecular biological approaches have been applied to study the genetic diversity of microorganisms, especially based on the analysis of 16S rDNA gene sequence. Therefore, this study is aimed at understanding the diversities of protease-producing *Bacillus* spp. used in tempeh production based on the 16S ribosomal RNA (rRNA) gene sequence. The results obtained will be further used as a base for further analysis of the role of protease-producing *Bacillus* strain in determining the quality of the tempeh in the future.

2. Materials and Methods

2.1. Screening of *Bacillus* spp. isolates from tempeh

Twenty-two tempeh samples were purchased from different tempeh producers in Jakarta, Bogor, Tangerang, on Java Island, Indonesia. Representative 25-g portions of tempeh were homogenized in 225-mL sterile 0.85% w/v NaCl by the use of a Stomacher lab-blender 400 (Seward Medical, London, UK) for 1 minute at "normal" speed. Appropriate decimal dilutions (0.1 mL) of the homogenates in the same diluent were spread over plates of plate count agar (PCA; Oxoid, England) after heating at 80°C for 10 minutes. Each plate was incubated at 37°C for 24 hours. All suspected protease-producing *Bacillus* spp. isolates were cultured on the PCA plates and incubated at 37°C for 24 hours. Gram positive, rod-shape cells, and endospore formation were the morphological characteristics used to determine the protease-producing *Bacillus* spp. All *Bacillus* spp. suspected isolates were stored at 4°C for further determination based on the sequence of 16S rRNA gene.

2.2. Preparation of genomic DNA

Bacterial cultures were grown overnight at 30°C in 50 mL of Luria-Bertani broth. Cells were recovered by centrifugation at 13,000×*g* for 3 minutes. Cell pellet was resuspended in a solution containing 1 mL of 10-mM Tris—HCl, pH 8.0, 10-mM EDTA, 100-mM NaCl, and 2% (w/v) SDS. Genomic DNA was isolated using a Genomic DNA Purification Kit (Fermentas, Lithuania) based on the manufacturer's protocol.

2.3. Amplification and DNA sequencing of 16S rRNA gene

Amplification of 16S rRNA genes of *Bacillus* spp. was performed in GeneAmp® PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA) using the primers 63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387R (5′-CAG GCC TAA CAC ATG CAA GTC-3′) (Marchesi *et al.* 1998). The PCR master mix (50 μL) contained 25-μL *GoTaq Green* (Promega, Madison, USA), 17-μL *Nuclease Free Water* (Promega, Madison, USA), 2 μL of each primer, and 4-μL DNA template (±100 ng). PCR conditions were as follows: predenaturation at 95°C for 5 minutes, denaturation at 95°C for 1 minute, annealing at 58°C for 5 minutes, extension at 72°C for 1 minute, and postextension at 72°C for 10 minutes. PCR products were observed using 1% electrophoresis agarose gel (Promega, Madison, USA) and then stained with ethidium bromide (Sigma Aldrich, USA). UV transilluminator was routinely used to visualize DNA in gel electrophoresis. PCR products were then partially sequenced in Macrogen Inc., Republic of Korea. The DNA sequencing results were aligned with 16S rRNA genes database provided by GenBank (www.ncbi.nlm.nih.gov) using *Basic Local Alignment Search Tool* (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree was constructed using MEGA 4.02 software (Tamura *et al.* 2007). Neighbour joining method was used to develop the phylogenetic tree.

2.4. Proteolytic assay

To determine the proteolytic activity, each *Bacillus* spp. isolate was inoculated onto Skim Milk Agar (2% skim milk + 1% bacto agar) then incubated at 37°C for 24 hours (duplo). Skim milk agar compositions used in this research was a modification of media used by Dajanta *et al.* 2009. Proteolytic activities were shown in the presence of a clear zone surrounding the bacterial colony and were determined by measuring the diameter of the clear zone.

3. Results

3.1. The presence protease-producing *Bacillus* spp. in tempeh

A total of 52 protease-producing *Bacillus* spp. had been isolated from Indonesian fresh tempeh. A total of 22 of 24 fresh tempeh samples were taken directly from the producers in Jakarta, Bogor, and Tangerang were found to be positive for protease-producing *Bacillus* spp. Four different types of *Bacillus* spp. morphology in tempeh were observed and were categorized as flower-like edges resembling a colony of mold, uneven edges, flat edges resembling cotton, and coral/rooted-like edges (Figure). The protease activity of each *Bacillus* spp. isolate highly varied, ranging from 8 to 26 mm after 24 hours incubation at 37°C (Table).

3.2. PCR amplification of 16S rRNA gene sequences dan phylogenetic profile

The genomes of all protease-producing *Bacillus* spp. isolates have been successfully isolated from the cell cultures using the protocol kit of Fermentas® Genomic DNA Purification Kit. PCR amplification of 16S rRNA gene sequences yielded DNA fragments

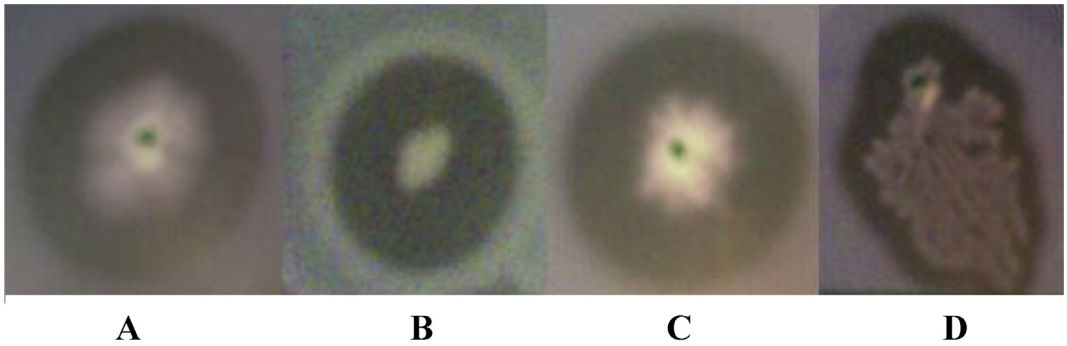

Figure 1. Four different types of protease-producing *Bacillus* spp. morphology from Indonesian fresh tempeh. A: flower-like edge resembling a colony of mold. B: uneven edges. C: flat edges resembling cotton. D: coral/rooted-like edges.

Table. The code, GeneBank accession number, name, percentage similarity, and proteolytic activity of protease-producing *Bacillus* spp. isolates from Indonesian fresh tempeh

Isolate code	GenBank accession number	References strain (GenBank)	Simila rity (%)	Proteolytic activity (mm)
E23	JF895956	*B. subtilis* strain 2G-1 (FJ493039.1)	99	26
ER2	JF895932	*B. cereus* strain C11C (HQ388817.1)	99	15
L15	JF895959	*B. subtilis* strain GB11 (EF101726.1)	99	15
B1a	JF895960	*B. pumilus* strain KD3 (EU500930.1)	97	14
B1d	JF895961	*B. pumilus* strain TBD3-1 (HQ236075.1)	99	11
EKA	JF895947	*B. cereus* isolate PGOa4 (EU162012.1)	99	19
EKC	JF895948	*B. cereus* strain SBD1-8 (HQ236038.1)	99	19
EKF	JF895949	*B. cereus* strain X-1 (HQ400407.1)	99	18
AB1	JF895950	*B. cereus* strain SBD2-1 (HQ236041.1)	99	16
ER4	JF895933	*B. megaterium* strain HDYM-24 (EF428248.2)	98	13
EK5	JF895946	*Bacillus* sp. strain LT3 (FJ946999.1)	99	18
GRQ	JF895942	*B. thuringiensis* strain BL-40 (HQ180398.1)	99	16
GR8	JF895943	*B. cereus* strain B1 (HM989918.1)	99	17
GRA	JF895944	*B. cereus* strain GL31 (EU418712.1)	98	19
EK4	JF895945	*B. cereus* strain S09053 (HQ224510.1)	99	17
B4c	JF895965	*B. pumilus* strain SBG2-4 (HQ236054.1)	99	11
B4d	JF895966	*B. cereus* strain ANctcri1 (HQ286640.1)	99	16
B4g	JF895967	*B. cereus* strain TAT3-7 (HQ236071.1)	98	17
B5e	JF895968	*B. pumilus* strain TBD3-1 (HQ236075.1)	99	8
ERE	JF895936	*Bacillus* sp. strain LT3 (FJ946999.1)	98	18
T2f	JF895976	*B. pumilus* strain TBD3-1 (HQ236075.1)	96	13
T3e	JF895977	*B. pumilus* strain TBD3-1 (HQ236075.1)	99	12
T3g	JF895978	*B. pumilus* strain SBG2-4 (HQ236054.1)	98	13
ERB	JF895934	*B. cereus* strain IMAUB1022 CS-2010 (HM752769.1)	99	16
ERD	JF895935	*B. cereus* isolate PGOa4 (EU162012.1)	98	18
GRH	JF895937	*B. cereus* isolate PGO6 (EU161996.1)	98	17
GRI	JF895938	*B. cereus* strain HMT6 (HQ156459.1)	99	18
GR9	JF895939	*B. cereus* strain 1BJ9 (HM161852.1)	99	19
GR1	JF895940	*B. cereus* strain HMT6 (HQ156459.1)	99	17
GRL	JF895941	*B. cereus* strain IMAUB1030 (FJ641013.1)	99	19
E22	JF895955	*B. amyloliquefaciens* strain 96XG3Y (FJ174650.1)	99	16
B6a	JF895969	*B. licheniformis* (AB513628.1)	98	12
B6c	JF895970	*Br. brevis* strain H2 (HM449127.1)	99	15
T1b	JF895973	*B. pumilus* strain BM4 (EU939702.1)	99	11
T1c	JF895974	*B. pumilus* strain TBD3-1 (HQ236075.1)	99	13
AB3	JF895951	*B. cereus* isolate LBS5 (EU400647.1)	99	18
AB4	JF895952	*B. thuringiensis* strain JMC-UBL 03 (HM451439.1)	100	19
ABB	JF895953	*B. cereus* strain DC3 (GQ344805.1)	99	19
ABE	JF895954	*B. cereus* strain B3 (HM989917.1)	99	17
B2a	JF895962	*B. cereus* strain X-1 (HQ400407.1)	99	16
B2c	JF895963	*B. cereus* strain TAT3-7 (HQ236071.1)	98	17
B3c	JF895964	*B. cereus* strain TBD3-2 (HQ236076.1)	100	18
B7c	JF895971	*B. cereus* strain HMT6 (HQ156459.1)	99	18
B10a	JF895972	*B. cereus* isolat PGOa4 (EU162012.1)	97	21
T2a	JF895975	*B. cereus* strain PGOa4 (EU162012.1)	99	17
T4b	JF895979	*B. pumilus* strain TBD3-1 (HQ236075.1)	99	13
T4d	JF895980	*B. cereus* strain C11C (HQ388817.1)	99	18
T5c	JF895981	*B. cereus* strain C11C (HQ388817.1)	99	17
T5e	JF895982	*B. cereus* strain ANctcri1 (HQ286640.1)	99	18
T6f	JF895983	*B. cereus* strain DS1 (HM773424.1)	99	19
T6g	JF895984	*B. cereus* strain TBD2-1 (HQ236074.1)	99	18
T6h	JF895985	*B. cereus* strain X-1 (HQ400407.1)	99	19

with single band at 1,300 bp for each *Bacillus* sp. strains (Figure 2 as representation).

BLASTN results of the partial sequence of 16S rRNA gene (about 800–950 nucleotides) showed high similarity with *Bacillus* spp.

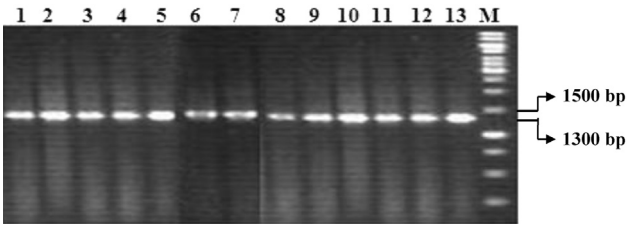

Figure 2. PCR amplification sequences of 16S rDNA of protease-producing *Bacillus* spp. from Indonesian fresh tempeh (1–13). Marker 1-kb ladder (M).

with maximum identities for each isolate in the range of 94%–100% with E-value 0 (Table). The DNA sequences of the 52 selected protease-producing *Bacillus* spp. have been deposited in GenBank with accession numbers JF895932–JF895956 and JF895959–JF895985 (Table). The distribution of all isolates was between the nine *Bacillus* spp. namely *B. cereus*, *B. pumilus*, *B. subtilis*, *B. thuringiensis*, *B. megaterium*, *B. licheniformis*, *Brevibacillus brevis*, *B. amyloliquefaciens*, and *Bacillus* sp. (Table).

A neighbour joining tree based on the 16S rRNA gene sequence of protease-producing *Bacillus* spp. had been successfully constructed. The phylogenetic tree showed that the 52 protease-producing *Bacillus* spp. isolates from tempeh were divided into two large clusters. The first cluster consisted of *B. cereus*, *B. thuringiensis*, *Bacillus* sp., and *B. brevis*. The second cluster consisted of *B. pumilus*, *B. subtilis*, *B. megaterium*, *B. licheniformis*, and *B. amyloliquefaciens* (Figure 3).

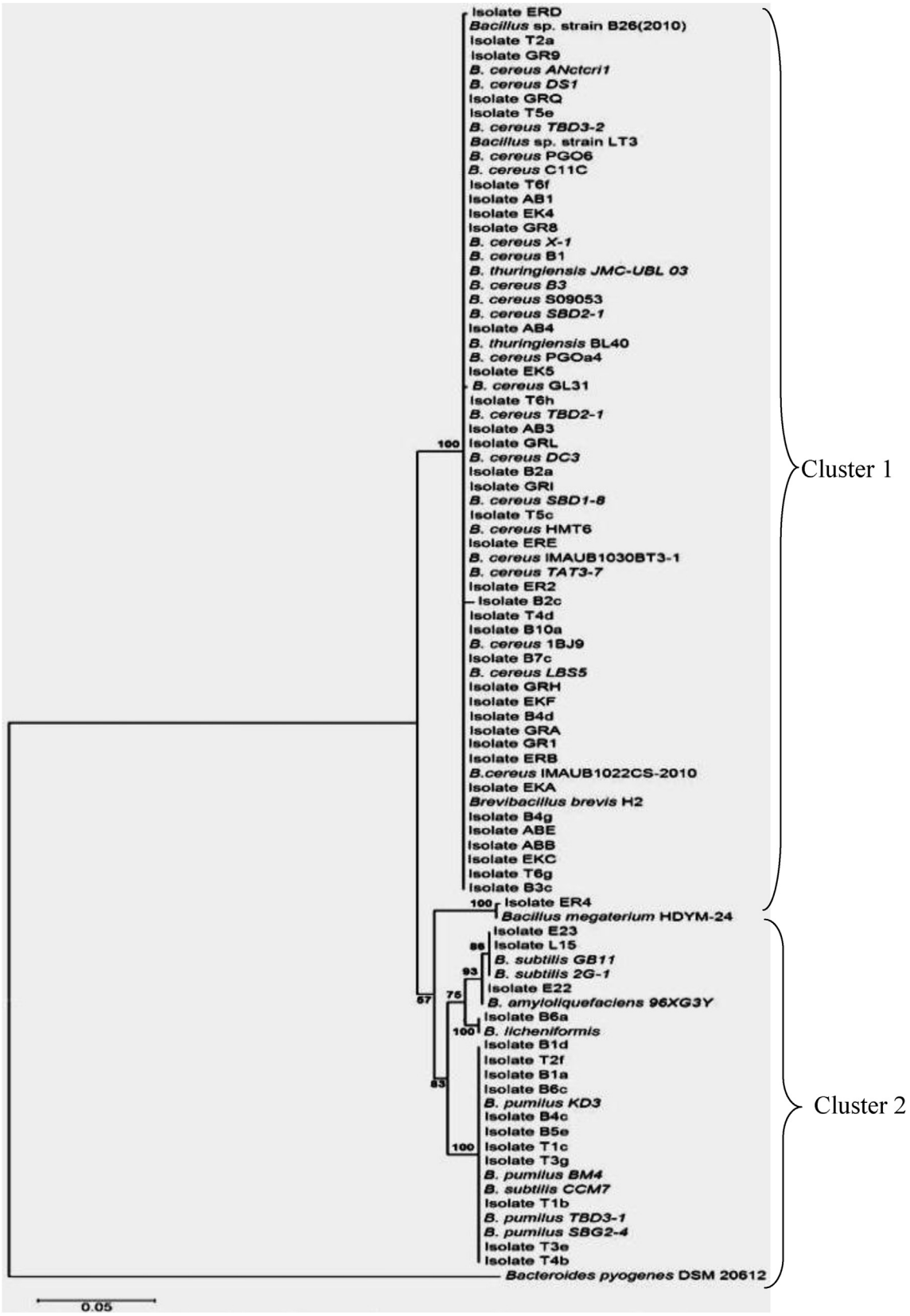

Figure 3. The phylogenetic tree of protease-producing *Bacillus* spp. from Indonesian fresh tempeh.

4. Discussions

In the fermentation of Indonesian tempeh, the quality of the final product is determined by the technology and quality of substrates used and the microorganisms involved during the fermentation process. It is widely known that the main microorganism in tempeh fermentation is *Rhizopus* spp. However, the results of this study showed that 22 Indonesian fresh tempeh were found to be positive for protease-producing *Bacillus* sp. This result supports the results of the study on the existence of *Bacillus* spp in fresh tempeh (Barus *et al.* 2008; Barus *et al.* 2010) that have been reported previously.

Based on the BLASTN results of 16S rRNA gene, *Bacillus* spp. was identified as *B. pumilus*, *B. subtilis*, *B. megaterium*, *B. licheniformis*, *B. cereus*, *B. thuringiensis*, *B. amyloliquefaciens B. brevis*, and *Bacillus* sp. *Bacillus* spp. are an important starter culture for alkaline food fermentations, such as Dawadawa (Amoa-Awua *et al.* 2006), chungkukjangs (Lee *et al.* 2007), kinema, and soumbala (Sarkar, *et al.*, 2002). The potential of *B. circulans*, *B. brevis*, *B. licheniformis*, *B. arabitane*, *B. coagulans*, *B. stearothermophillus*, and *B. subtilis* is diverse in determining the quality of chungkukjang [a traditional Korean food made of fermented soybeans (Lee *et al.* 2007)]. However, information about the *Bacillus* spp. role in determining the quality of tempeh has not been reported and this is a preliminary study of *Bacillus* spp. from tempeh.

Tempeh is one of the important sources of protein for Indonesians. The survey results indicate that each person on average consumes tempeh three times a week (data not shown). Therefore, to increase the digestibility of soy proteins, the use of microorganisms with proteolytic activity that can digest soy proteins during tempeh fermentation process is necessary. All *Bacillus* spp. isolated in this study had proteolytic activity (Table). The protease activity of each *Bacillus* spp. isolate highly varied, ranging from 8 to 26 mm after 24 hours incubation at 37°C. It was found that *B. subtilis* (isolate E23) has the highest proteolytic activity (26 mm). Digestibility of soya bean fermented with *Bacillus* spp. was reported by Kiers *et al.* 2000. The role of *Bacillus* spp. in improving the quality of fermented soybeans has also been widely reported such as Natto (Wei *et al.* 2001; Wei and Chang 2004), kinema (Sarkar and Tamang 1995; Tamang and Nikkuni 1996) and soy-daddawa (Omafuvbe *et al.* 2002; Omafuvbe 2008), and thua nao (Leejeerajumnean, 2003). Therefore, the results of this investigation are important as a basis for the selection of *Bacillus* spp. for further investigation of its role in determining the quality of tempeh.

The process of tempeh making in Indonesia is still using conventional methods with uncontrolled condition (Barus *et al.* 2008). During the fermentation process, not only fungi are involved but also bacteria. Tempeh produced in Indonesia naturally harbors *K. pneumoniae* and *Escherichia coli*. Some strains of *E. coli* and *K. pneumoniae* could induce diseases such as diarrhea and pneumonia. However, it has been reported that *K. pneumoniae* and *E. coli* from Indonesian tempeh were genetically different from that of the pathogenic isolates (Ayu *et al.* 2014; A'yun *et al.* 2015). In this study, we also found *Bacillus cereus*. It has already been known that *B. cereus* is a bacterium that can cause food spoilage and diarrhea (Ultee *et al.* 1999). The presence of *Bacillus cereus* in traditional fermented food has been widely reported, such as in "*okpehe*", kinema, and soumbala (Sarkar *et al.* 2002). However, based on the data, poisoning caused by *Bacillus* spp. from consuming tempeh has never been reported elsewhere. This may be due to several factors such as cooking before consumption or that those strains of *B. cereus* found in tempeh might be harmless. Therefore study of *B. cereus* pathogenic characteristic is still important to study.

Phylogenetic tree (Figure 3) showed that fifty-two protease-producing *Bacillus* spp. isolates from tempeh were divided into two large clusters. The first cluster consisted of *B. cereus*, *B. thuringiensis*, *Bacillus* sp. and *B. brevis*. The second cluster consisted of *B. pumilus*, *B. subtilis*, *B. megaterium*, *B. licheniformis*, and *B. amyloliquefaciens* (Figure 3).

Bacillus in tempeh is diverse. However, intraspecies differentiation of the *Bacillus* isolates E23 (*B. Subtilis*) and L15 (*B. Subtilis*) and *Bacillus pumilus* among isolates B1a, B1d, B4c, B5e, T1b, T1c, T2f, T3e, T3g, and T4b based on the phylogenetic tree cannot be achieved. Thus, other molecular methods are needed for intraspecies differentiation of the *Bacillus* spp.

Conflict of Interest

The authors declare no conflict of interest. All experiments in this study comply with the current laws of the country where they were performed.

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